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BIOSYNTHESIS AND PURIFICATION OF V AND W ANTIGENS IN PASTEURELLA PESTIS

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ABSTRACT

The separation and purification of V and W antigens are described. The methods that gave the best results were:

(a) The precipitation of both antigens from the supernatant fluid of a 36°C-grown culture of strain M23 by use of ammonium sulfate.

(b) Chromatography on DEAE cellulose.

V antigen was eluted with 0.1 M NaCl and W antigen with 0.5 M NaCl. Recycling both antigens on DEAE cellulose resulted in a sample containing approximately 20 units of V antigen per milligram of protein (100-fold purification) and no W antigen, and a sample containing 600 units of W antigen per milligram of protein (1000-fold purification) and no V antigen.

V antigen is a protein with a molecular weight of 90,000 and W antigen is a lipoprotein with a molecular weight of 145,000.

Both antigens were stable at 60°C, but not at 80°C, for 30 minutes. W antigen, but not V, was lost upon extensive dialysis against distilled water or pervaporation. Both antigens were reduced in titer by prolonged storage at 5°C or by lyophilization, but not by storage at -20°C.

Based on the use of rabbit antisera containing only V antibody or only W antibody, the conclusion was drawn that V antibody, but not W antibody, can protect mice against plague.

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TABLES

I. INTRODUCTION*

Studies by Burrows and Bacon demonstrated that virulent strains of Pasteurella pestis[1],[2] and Pasteurella pseudotuberculosis[3] produce two antigens, designated V and W, that are not produced by most avirulent strains. Biological effects associated with these antigens, as observed by use of live V^+W^+ and V^-W^- strains, indicated that the antigens were associated with resistance to phagocytosis[4] and were essential for the best live vaccine in mice.[5] Direct evidence on the activity of each antigen depends on their separation and purification. This manuscript summerizes our investigation into the biosynthesis, separation, purification, and protective value of V and W antigens.

II. MATERIALS AND METHODS

A. STRAINS

P. pestis strain M-23[6] was chosen because it appeared to produce as much V and W antigens as any other virulent strain, but did not produce detectable amounts of Fraction I, a protein-carbohydrate complex usually present in large quantities in the supernatant fluid of typical virulent strains of P. pestis. P. pestis strain Alexander was used to challenge animals in passive protection experiments.

B. QUANTITATION OF ANTIGENS

V and W antigens were assayed by diffusing various dilutions of a sample against a standard V or W antiserum in a gel plate under standard conditions.[7] The greatest dilution that showed a visible band of precipitate was considered to contain one unit of antigen per milliliter. Purification results are expressed in terms of units of antigen per milligram of protein, and the minimum number of antigens associated with a sample as determined by diffusion against complex antisera.

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* "In conducting the research reported herein, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

C. CHEMICAL AND PHYSICAL ASSAYS

Protein was measured by either the spectrophotometric method, which relates protein concentration to the absorption of light at 215 millimicron,[8] or the chemical method of Lowry.[9] Carbohydrate was measured by the diphenylamine method.[10] Lipid was estimated by the method of Snyder and Stephens.[11] Nucleic acid was calculated from the absorption of light at 260 millimicrons. Molecular weights were calculated by the gel diffusion method of Polson.[12]

III. RESULTS

V and W antigens are produced at 37°C but not at 26°C.[2] However, at 37°C, avirulent V^-W^- mutants are favored in many media, resulting in little or no V and W antigen production. To avoid this, strain M-23 was grown at 26°C in Difco heart infusion broth for 24 hours and the cells were sedimented and resuspended at 36°C for 16 hours under various conditions. This study resulted in the following conclusions:

(a) At least three per cent Bacto Casitone was necessary for the synthesis of V and W antigens.

(b) Under these conditions, at least 0.01 M sodium gluconate was essential. Ammonium gluconate was equally effective, and magnesium gluconate doubled the production of V and W antigens. It has been demonstrated by Brubaker and Surgalla* that, under different cultural conditions, energy sources other than gluconate can support the production of V and W antigens.

(c) Calcium inhibited the synthesis of V and W antigens. This observation has been confirmed and extended by Brubaker and Surgalla.*

(d) No synthesis of V or W antigens occurred in an atmosphere of 100 per cent CO_2, of 95 per cent N_2 plus 5 per cent CO_2, or *in vacuo*.

A. PRODUCTION OF ANTIGENS FOR PURIFICATION

On the basis of these findings, strain M-23 was grown in Difco heart infusion broth at 26°C. The cells were sedimented, resuspended in a medium (300 milliliters per two-liter flask) of 5 per cent Bacto Casitone, 0.04 sodium gluconate, and 0.04 M magnesium sulfate, and shaken at 36°C for 16 hours. During the 16-hour incubation period, the viable number of microorganisms remained at $1x10^9$ to $2x10^9$ per milliliter, and the pH

* Manuscript in preparation

remained near 7.0. The contents of the flasks were pooled and the cells permitted to settle overnight at 5°C. The clear supernatant fluid was siphoned off, filtered through sintered glass, and brought to 3.0 M with solid ammonium sulfate. The precipitated proteins were collected after standing approximately one-half hour, pH 6, 5°C, resuspended in a minimum amount of distilled water and dialyzed overnight against cold distilled water.

A comparison of the supernatant fluid with the cell contents (obtained by sonication or acetone drying) indicated that the total amount of V and W antigens was split about 50:50 between the cells and the supernatant fluid, but the units of each antigen per milligram of protein were twice as much in the supernatant fluid. In addition, there was a much higher concentration of nucleic acid in the disrupted cells. Precipitation with 3.0 M ammonium sulfate and subsequent dialysis resulted in a tenfold concentration and a fourfold-to fivefold purification of both antigens.

B. PURIFICATION

1. Ammonium Sulfate Fractionation

A sample of approximately 400 milliliters of sterile supernatant fluid that had been concentrated by ammonium sulfate and dialyzed was used to determine the effectiveness of fractional increases of ammonium sulfate on the purification of V and W antigens. A ten-milliliter sample (1.1 units V and W antigens per milligram of protein) was brought to 1.2 M by the addition of 3.5 M ammonium sulfate, pH 7.0, held ten minutes, and centrifuged at 27,000 x g for five minutes. The precipitate was resuspended in distilled water and tested for V, W, and total protein. The supernatant fluid was brought to 1.4 M, and the procedure was repeated until 2.2 M ammonium sulfate was reached. The results (Table I) indicated that both V and W antigens were precipitated between 1.2 and 2.2 M, with the maximum purification occurring at 1.8 M (sixfold purification).

This experiment was repeated with the exception that each additional increment of ammonium sulfate was allowed to equilibrate with the sample overnight at 5°C. The results were essentially the same as those obtained with ten-minute equilibration.

The remainder of the 400-milliliter batch was brought to 1.4 M ammonium sulfate, the precipitate removed, and the supernatant fluid brought to 2.2 M ammonium sulfate. The results indicated that 63 per cent of both V and W antigens were recovered in the 2.2 M precipitate with a 4.5-fold purification. Since considerable quantities of V and W were precipitated at 1.4 M, subsequent batches were handled by taking the material that precipitated between 1.3 and 2.2 M ammonium sulfate.

TABLE I.

FRACTIONATION OF V AND W ANTIGENS WITH AMMONIUM SULFATE (10-MINUTE EQUILIBRATION)

Molarity [a] of ammonium sulfate	V		W		Total protein mg
	Total units	Units/mg protein	Total units	Units/mg protein	
1.2	20	0.3	10	0.1	67.0
1.4	20	0.8	40	1.5	26.0
1.6	40	2.6	80	5.2	15.2
1.8	80	6.7	80	6.7	12.0
2.0	80	3.6	80	3.6	22.5
2.2	20	2.5	20	2.5	8.0
(2.2 supernatant fluid)	0	0	0	0	72.2
Totals	260		310		223
Original material	320	1.1	320	1.1	296

a. Original material was brought to the molarity indicated by the addition of 3.5 M ammonium sulfate, pH 7.0.

2. Cellulose Chromatography

The wide application of various forms of cellulose for the separation of proteins suggested this method for the purification of V and W antigens. Initially, ten-milliliter samples of material containing V and W antigens (precipitated between 1.3 and 2.2 M ammonium sulfate) were adsorbed on one gram of the following materials: (a) cellulose phosphate, (b) carboxymethylcellulose, (c) aminoethylcellulose, and (d) diethylaminoethylcellulose. Only the diethylaminoethyl (DEAE) cellulose adsorbed all of the V and W antigens, and this material was used for all subsequent cellulose fractionation.

In order to observe the behavior of V and W antigens on DEAE cellulose, 46 different runs were performed. These experiments involved many variables, such as the amount of protein per gram of cellulose required to saturate a column, the resolution obtainable with a stepwise elution versus a gradient elution, the use of regenerated cellulose compared with unused cellulose, etc. An over-all view of the behavior of these antigens on DEAE cellulose is shown in Table II.

TABLE II.

SUMMARY OF TYPICAL DATA OBTAINED ON THE FRACTIONATION OF V AND W ANTIGENS ON DEAE CELLULOSE

Experiment No.	Antigen [a]	Units/mg Protein		% Recovery
		Before	After	
1	V	1.1	3.0	12
	W	2.2	12.3	14
2	V	0.8	5.8	63
	W	3.2	182	13
W recycled	W	182	291	100
3	W	1.3	32	7
W recycled	W	32	620	100
4	V	0.9	2.1	40
	W	0.7	73	60
5	V	1.0	2.7	5
	W	2.1	22	26

a. In all experiments shown, V antigen was eluted with 0.1 M NaCl and W antigen with 0.5 M NaCl.

These data are based on similar but not identical experiments. We found that the greatest adsorption of V antigen occurred when the sample was dialyzed against distilled water and then placed on DEAE cellulose that had been washed with 1 N NaOH, neutralized with 0.018 M potassium phosphate buffer, pH 6.8, and thoroughly rinsed with distilled water. W antigen was adsorbed more than V antigen and, therefore, presented no problem in processing large quantities. The difference in the percentage recovered of V antigen may be attributed to oversaturating the column in some cases.

The elution schedule used routinely was to wash the column stepwise with distilled water, 0.1 M, 0.3 M, and 0.5 M NaCl. The eluate flowed through a silica cell and the amount of protein it contained was estimated with an improvised recording spectrophotometer consisting of a Bausch and Lomb monochromatic light source (set at 280 millimicron) and a photosensitive tube connected to a Brown recorder. Each fraction was eluted until the recorder indicated that the eluate contained no protein. Depending on the amount of protein per gram of cellulose, a portion of the V antigen, but never W antigen, could be found in the distilled water wash. All the V antigen that adsorbed to the column, but none of the W antigen, was eluted with 0.1 M NaCl. The 0.3 M NaCl eluate contained a large amount of protein, but no V antigen, and usually little or no W antigen. The 0.5 M NaCl eluate contained little protein, but most of the W antigen.

The use of DEAE cellulose for the purification of W antigen, as well as the separation of V and W antigens, was very effective. The average figure before cellulose chromatography was 1.0 unit of W per milligram of protein and 64 units of W per milligram of protein after cellulose chromatography. This latter figure was increased to as much as 620 by recycling the W antigen through a small DEAE cellulose column. Unlike V antigen, W antigen adsorbed strongly to cellulose, and even under conditions of high protein per gram of cellulose, all the W antigen was adsorbed. Its instability, to be discussed later, probably contributed to the poor recovery in some cases. By use of gel diffusion, it was demonstrated that only W antigen could be detected when the purified sample was diffused against several complex antisera.

The results obtained with DEAE cellulose for the purification of V antigen were not as good as those obtained with W antigen. In terms of units of V antigen per milligram of protein, the average value went from 1.0 before to 3.4 after cellulose chromatography. The percentage recovered was good only when the ratio of protein to cellulose was small. Processing large batches of V antigen on a 50-gram DEAE cellulose column resulted in most of the V antigen's being washed through with distilled water. For example, with 125 milligrams of protein per gram of cellulose, the recovery in the 0.1 M NaCl eluate was 63 per cent, but with 2800 milligrams of protein per gram of cellulose, the recovery in the 0.1 M NaCl eluate was only five per cent. By use of gel diffusion, a minimum of five to seven antigens were shown to be still present in the 0.1 M eluate. Recent experiments, in which the 0.1 M eluate containing V antigen was recycled on DEAE cellulose and eluted stepwise with 0.06, 0.08, and 0.10 M NaCl, have yielded samples containing 20 units of V antigen per milligram of protein with fairly good recovery.

3. Other Purification Methods

Several different purification possibilities were investigated but discontinued because of their lack of promise. Included among these were calcium phosphate chromatography, continuous flow curtain electrophoresis, precipitation by cold methanol, acid precipitation, Zn^{++} precipitation, fractional solubilization of an ammonium sulfate precipitate by a gradient from 2.5 M ammonium sulfate to distilled water, chromatography on G25 Sephadex, and elution from specific antigen-antibody precipitates. None of these methods improved on the results obtained with ammonium sulfate precipitation and DEAE cellulose chromatography.

C. STABILITY OF V AND W ANTIGENS

Both V and W antigens were detected qualitatively after heating purified samples for 30 minutes at 40°C and 60°C, but not at 80°C or 100°C.

When 25 milliliters of a culture supernatant fluid were placed in a dialysis bag (45-millimeter diameter) and dialyzed against 400 milliliters of distilled water at 5°C with constant agitation by a magnetic stirrer, all the W antigen, but none of the V antigen, was lost after two days. During the two-day dialysis period, the protein remained at 0.10 milligram per milliliter.

Repetition of this experiment again demonstrated the loss of W antigen. Ten milliliters of a fraction precipitated between 1.3 and 2.2 M ammonium sulfate and containing eight units of W antigen per milliliter were dialyzed against 250 milliliters of distilled water at 5°C with agitation. After one day, only four units of W per milliliter were present, and after four days no W antigen was detected. The 250 milliliters of dialysate were concentrated to ten milliliters and tested in a gel plate, but contained no detectable W antigen.

The titer of both V and W antigens gradually decreased during prolonged storage at 5°C. For example, one of the best batches produced had a titer of 12 units of V antigen and 24 units of W antigen per milliliter of supernatant fluid, but dropped to eight units of V and 16 units of W per milliliter after storage for three days at 5°C. Samples of a greater purity seemed more stable, but it was common to lose 50 per cent of the activity of both V and W antigens during storage for one to two weeks at 5°C. An example illustrating the effect of storage, dialysis, and lyophilization on a typical preparation of V and W antigens is shown in Table III.

The results of two lyophilization experiments demonstrated a 50 per cent loss of both V and W antigens. For this reason, lyophilization of materials was avoided.

TABLE III.

EFFECT OF STORAGE, DIALYSIS, AND LYOPHILIZATION ON A TYPICAL PREPARATION OF V AND W ANTIGENS

Sample	Total Units of Antigen	
	V	W
(1) Culture supernatant fluid	8400	66,500
(2) Same as (1), incubated at 5° for 3 days	6000	32,000
(3) Same as (2), dialyzed overnight	5800	11,200
(4) Same as (3), lyophilized and resuspended	3300	7600

On several occasions both crude and partially purified V and W antigens have been frozen at -20°C without loss of either antigen after thawing. This method is used routinely when a sample containing these antigens is to be held more than a few days.

In most of the methods used to purify V and W antigens, it is essential to concentrate the fractions obtained in order to detect by gel diffusion methods the antigens originally present. Since at least one-half of both V and W antigens were lost after lyophilization, other methods of concentrating proteins were tried. Five milliliters of a sample containing eight units of V and eight units of W antigens per milliliter were diluted to 100 milliliters with distilled water and pervaporated in a cellophane sac in front of a large fan until only five milliliters remained. Assay of the material after concentration by this method indicated that 100 per cent of the V antigen, but only 25 per cent of the W antigen, was recovered. Pervaporation was used subsequently to concentrate samples containing V antigen. The method finally adopted for the concentration of W antigen was to place the sample in a cellophane sac and surround the sac with Carbowax (polyethylene glycol compound 20-M).* This material draws water, but not protein, out of the sac. By this method, a sample could be held at 5°C and be concentrated 10 to 100 times with no loss of W antigen.

D. PHYSICAL AND CHEMICAL ANALYSES

Three separate molecular weight determinations for each antigen were within the range of 85,000 to 95,000 for V antigen and 140,000 to 150,000 for W antigen. On a dry-weight basis, the best V antigen preparation contained less than one per cent carbohydrate and nucleic acid and 100 per

* Carbide and Carbon Chemicals Company, New York 17, N. Y.

cent protein. The best W antigen preparation contained 38 per cent lipid and 59 per cent protein. Other purified W antigen preparations contained less than five per cent carbohydrate. The lipoprotein nature of W antigen correlates with the recent finding that W antigen (M.W. = 145,000) appeared less dense than V antigen (M.W. = 90,000) when both antigens were sedimented in a sucrose density gradient.

Both V and W antigens were destroyed by trypsin, as judged by loss of ability to react with specific antisera in a gel plate.

E. IMMUNIZATION OF ANIMALS

Injection of either purified V or W antigens mixed with Freund's adjuvant did not induce the formation of detectable antibodies in either mice or guinea pigs, but did induce antibody production in rabbits. However, recent experiments employing Algivant* as an adjuvant resulted in the production of both V antibody and W antibody in guinea pigs when 100 units (equivalent to approximately 100 micrograms) of either antigen were injected intramuscularly in two equal doses spaced two weeks apart. Although the groups of animals were small, the result of challenging these actively immunized guinea pigs indicated that V antigen, but not W antigen, was protective. This result was confirmed by passive immunization studies employing rabbit serum containing either V antibody or W antibody (Table IV). Rabbit anti-V serum injected into the peritoneal cavity of mice concomitantly with a 100^2 LD_{50} dose of virulent *P. pestis* strain Alexander protected all the mice. Rabbit anti-W serum or normal rabbit serum showed no protective effect under the same conditions.

IV. DISCUSSION

One of the conclusions to be drawn from this work is the apparent importance of V antibody and the lack of importance of W antibody in protection against plague. The lack of passive protection of W antibody in mice (Table IV) supports the data of Burrows and Bacon[5] that guinea pig antiserum containing measurable amounts of W antibody was nonprotective against *P. pestis* strain M-23 challenge in mice.

By use of live purine-dependent strains as vaccines, Burrows and Bacon[5] found V antigen to be poorly antigenic in mice and nonantigenic in guinea pigs. Although our experience in vaccinating animals with purified V antigen supports the impression that V is a poor antigen, results with Algivant indicated that it can induce antibody formation even in guinea pigs. The apparent importance of V antibody would make an investigation into methods of increasing the antigenicity of V seem worthwhile.

*Colab Laboratories, Inc., Chicago Heights, Illinois

Unfortunately, V antigen was more difficult to purify than W antigen. One promising possibility of increasing the purity of V antigen is the use of density gradient centrifugation.

Although V and W antigens were always produced together (usually just twice as many units of W antigen as there were of V antigen), there was no evidence to support the idea expressed by Burrows and Bacon[5] that one antigen may be the precursor of the other. Culture supernatant fluids containing known amounts of each antigen never increased in the concentration of one antigen at the expense of the other.

TABLE IV.

PASSIVE PROTECTION OF MICE AGAINST PLAGUE WITH RABBIT ANTISERA CONTAINING V ANTIBODY OR W ANTIBODY

	Dead/Total Challenged[b]			
Rabbit serum[a]	Exp. 1	Exp. 2	Exp. 3	
Normal	10/10	10/10	10/10	
Anti-V[c]	0/10	-	0/5	
Anti-W[d]	-	10/10	5/5	5/5

a. Five-tenths milliliter of rabbit serum was injected into the peritoneal cavity concomitantly with the challenge dose. Three days later, another 0.5 milliliter of serum was injected via the same route.
b. Mice were challenged with approximately 1000 P. pestis strain Alexander (100 times the LD_{50} dose) via the intraperitoneal route.
c. Two different antisera were tested.
d. Three different antisera were tested.

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